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14. ABSTRACT

We hypothesize that targeted molecular intervention can preserve vision threatened by battlefield trauma-induced corneal and retinal inflammation, corneal and retina/optic nerve apoptosis, ocular surface dry eye after refractive surgery, and retinal degeneration. We are studying the consequences of trauma-induced (1) corneal inflammation using a gene therapy approach of providing soluble Fas ligand to the cornea to determine if this ligand can suppress corneal inflammation in mice; (2) retinal inflammation by examining if transforming growth factor-beta, thrombospondin, and somatostatin, in subretinal space, can suppress inflammation within retina secondary to autoimmune uveoretinitis and light-induced damage in mice; (3) corneal cell death by apoptosis and promote regeneration by identifying the anti-apoptotic gene with the greatest capacity to suppress corneal cell apoptosis using mice; (4) retinal cell death and regeneration by using mice to determine if systemic treatment with lithium chloride can prevent collateral damage to retinal neurons and promote optic nerve regeneration; (5) dry eye by determining how to minimize dry eye after LASIK refractive surgery by developing new tests to predict pre-disposition to refractive surgery induced dry eye; and (6) retinal injury by generating stem cell polymer composites.

15. SUBJECT TERMS
blindness, trauma, eye, cornea, retina, dry eye, refractive surgery, inflammation, optic nerve, regeneration

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Introduction

An increasing percentage of battlefield injuries occur to the eye in modern warfare. Even treatable battlefield injuries to the eye can lead to blindness because of collateral damage to adjacent tissues. This blindness results from injury-induced inflammation, cell death, failure to regenerate and repair, and development of scar tissue. Task #5 is one portion of a multidisciplinary project that addressed corneal blindness resulting from abrasions, burns, and penetrating wounds acting on normal corneas or exaggerated in corneas that have undergone refractive surgery, as well as retinal blindness resulting from physical trauma, infection, or laser-induced injury that destroy retinal nerve cells. In task 5 our goal is to prevent the consequences of trauma to the cornea after refractive surgery by developing strategies to diagnose dry eye syndromes. Our specific objective was to determine if there are individuals in whom the goblet cells of the conjunctiva do not respond normally to neural and growth factor stimulation and if this abnormal response predisposes these individuals to developing chronic dry eye after laser refractive surgery. Our three subtasks were to 1: determine if the response of conjunctival goblet cells to nerves and growth factors is reduced in a mouse model of dry eye and if loss of corneal nerves (induced by a corneal wound) alters this response. The loss of corneal nerves by a corneal wound mimics the loss of nerves induced in laser refractive surgery. 2: determine if human goblet cells from normal human controls respond to the growth factor EGF, the b-adrenergic agonist isoproterenol, and the cholinergic agonist carbachol. 3: Determine if patients with reduced goblet cell response will have an increased rate of dry eye symptoms and traumatic complications after laser refractive surgery.

Body

I. Research accomplishments for Subtask 1: We have finished our studies on measuring alterations in phosphoprotein levels in conjunctiva of Balb-c mice following stimulation with EGF, carbachol and isoproterenol. We performed corneal wounding (superficial keratectomy) in 12 week-old Balb-c mice in order to mimic laser refractive surgery. Corneal wounding was done in the right eye and left eye was kept unwounded. Mice not wounded in either eye were considered as the controls. The conjunctiva was isolated from both eyes at different time points (2 days, 6 days, 2 weeks and 4 weeks) following wounding and divided into four pieces. Long time points were chosen as our goal is to study chronic dry eye that develops after surgery in a percentage of individuals rather than the acute dry eye that occurs in almost everyone after this surgery. Conjunctival tissue pieces were stimulated with no additions (basal), EGF (10^{-7} M,) carbachol (10^{5} M), and isoproterenol (10^{-5} M) for 5 minutes at 37° C in a water bath. The reaction was stopped in keratinocyte basal medium kept at 4° C. The conjunctival lysates were prepared by homogenizing the tissue in RIPA buffer. BioRad multiplex assay was done to study the levels of various phosphoproteins in the conjunctival lysate samples and to determine alterations in the phosphoprotein levels following corneal wounding. Seven-plex assay kit was used to measure the levels of phosphorylated ERK (p42/p44 mitogen-activated protein kinase), JNK, p38 mitogen activated protein kinase, AKT, IkB alpha, STAT-3 and P70S6 at the same time in each sample. Phosphoproteins levels were standardized to the amount of total ERK (phosphorylated and non-phosphorylated) in each sample. The data were analyzed using Bioplex manager software and fold increase in phosphoprotein levels was determined over the basal levels. Statistical analysis was done using student T test and $P < 0.05$ was considered as significant.

A. Results from non-wounded control mice:

Table 1 summarizes the results from 12 week-old control (n=7) mice. We found:

1. Significant increase in phosphorylated ERK following stimulation with EGF and carbachol.
2. Significant increase in phosphorylated AKT following stimulation with EGF and carbachol.
3. Significant increase in STAT-3 following stimulation with isoproterenol.

4. Decrease in the levels of P70S6 were seen following stimulation with isoproterenol, although it was not statistically significant.

B. Results from the wounded (right) eye 2 days following corneal wounding :

Table 2 summarizes the results in the wounded eye 2 days after superficial keratectomy performed in 12 weeks old Balb-c mice (n=5). We found:
1. Significant increase in phosphorylated ERK following stimulation with EGF and carbachol.
2. Significant increase in phosphorylated JNK following stimulation with EGF and carbachol.
3. Significant increase in phosphorylated P38 MAPK following stimulation with EGF.

C. Results from the unwounded (left) eye 2 days following corneal wounding :

Table 3 summarizes the results in the unwounded eye 2 days after superficial keratectomy performed in 12 weeks old Balb-c mice (n=5). We found:
1. Significant increase in levels of phosphorylated ERK following stimulation with EGF.
2. Significant increase in phosphorylated JNK following stimulation with carbachol.
3. Significant increase in phosphorylated P38 MAPK following stimulation with EGF and carbachol.
4. Significant increase in phosphorylated AKT following stimulation with isoproterenol.
5. Signicant increase in phophorylated IkB alpha, STAT-3 and P70S6 following stimulation with carbachol.

D. Results from the wounded (right) eye 6 days following corneal wounding

Table 4 summarizes the results in the wounded eye 6 days after superficial keratectomy performed in 12 -week old Balb-c mice (n=3). We found:
1. Significant decrease in phosphorylated p38MAPK following stimulation with EGF.
2. Significant decrease in phosphorylated AKT following stimulation with EGF.

E. Results from the unwounded (right) eye 6 days following corneal wounding

Table 5 summarizes the results in the wounded eye 6 days after superficial keratectomy performed in 12 -week old Balb-c mice (n=3). We found:
1. Significant increase in phosphorylated pERK following stimulation with EGF and carbachol.

F. Results from the wounded (right) eye 2 weeks following corneal wounding

Table 6 summarizes the results in the wounded eye 2 weeks after superficial keratectomy performed in 12 -week old Balb-c mice (n=6). We found:
1. Significant increase in phosphorylated pERK following stimulation with EGF.
2. Significant increase in phosphorylated pJNK following stimulation with EGF.
2. Significant increase in phosphorylated pAKT following stimulation with carbachol.

G. Results from the unwounded (right) eye 2 weeks following corneal wounding

Table 7 summarizes the results in the unwounded eye 6 days after superficial keratectomy performed in 12 -week old Balb-c mice (n=6). We found:
1. Significant decrease in phosphorylated p38MAPK following stimulation with carbachol.

H. Results from the wounded (right) eye 4 weeks following corneal wounding

Table 8 summarizes the results in the wounded eye 4 weeks after superficial keratectomy performed in 12 -week old Balb-c mice (n=6). We found:
1. Significant increase in phosphorylated pERK following stimulation with EGF.

I. Results from the unwounded (left) eye 4 weeks following corneal wounding

Table 9 summarizes the results in the unwounded eye 4 weeks after superficial keratectomy performed in 12 -week old Balb-c mice (n=6). We found:
1. Significant increase in phosphorylated pERK following stimulation with EGF.

J, Summary of completed experiments

Table 10 summarizes the number of completed experiments
We have done:

1. Seven stimulation experiments on unwounded mice and analyzed all seven with multiplex.
2. Five stimulation experiments on mice 2 days after wounding and analyzed all five with multiplex.
3. Four stimulation experiments on mice 6 days after wounding and analyzed three with multiplex. Samples from one mouse were unable to be analyzed.
4. Six stimulation experiments on mice 2 weeks after wounding and analyzed six with multiplex.
5. Six stimulation experiments on mice 4 weeks after wounding and analyzed six with multiplex.

II. Research Accomplishments for Subtask 2: As Dr. Dimitri Azar our initial collaborator moved from Massachusetts Eye and Ear Infirmary, we revised our IRB protocol to remove him as the doctor to whom adverse advents would be reported and replaced him with Dr. Reza Dana. Impression cytology samples were removed from 14 volunteers. Four samples were obtained from one eye. In the first patients we developed the method for collecting cells from the nitrocellulose membrane and found that placing the membrane in a glass centrifuge tube and centrifuging resulted in the best yield of cells and the most responsive cells as determined by western blotting analysis. Unfortunately not enough protein was recovered in each sample to perform the multiplex analysis. We thus decided to combine samples resulting in two final samples. These samples were stimulated by no additions and carbachol. Using this method we were not be able to study the effect of EGF and isoproterenol. This change was acceptable, as carbachol works via EGF and isoproterenol did not stimulate any phosphoprotein activity in the mouse model. We tried this in samples from one volunteer. Enough protein was obtained in each sample and carbachol increased pERK and p38 MAPK activity. Impression cytology samples were obtained from 10 volunteers at Walter Reed Army Medical Center, stimulated at that location, frozen on dry ice, and transported to the Schepens Eye Research Institute. In most samples there was not enough protein to measure by the Bradford Assay. In spite of this we performed the multiplex analysis. However, for most of the samples there was no consistent stimulation of phosphoprotein activity by carbachol and in most cases there was no stimulation. We felt that for the assay to work, the samples could not be frozen before analysis. As this was not possible, we terminated these experiments and changed the protocol for Subtask 3.

III. Research Accomplishments for Subtask 3: As Dr. Dimitri Azar our initial collaborator moved from Massachusetts Eye and Ear Infirmary, we enlisted COL Kraig S. Bower, LTC Charles Coe, and Ms. Denise Sediq from Walter Reed Army Medical Center as new collaborators. Our IRB documents were approved and the details of the clinical study specified. However, with the failure of Subtask 2, we changed portions of the clinical study. We decided to continue collecting impression cytology specimens, but instead of stimulating them, we would analyze them by quantifying the number of filled goblet cells per total number of goblet cells using immunofluorescence microscopy. In addition, we began to collaborate with Dr. Robert Sack of SUNY State College of Optometry, New York, NY. We will send Dr. Sack the Schirmer strips that we are already using to determine the volume of tears. He will analyze the tears collected on them by microarray for inflammatory mediators. We have new, approved protocols for these changes.

There were a total of 146 patients enrolled in the study. Eight patients were withdrawn. Three patients withdrew prior to treatment, one voluntarily switched to a different study, one was no longer eligible for surgery due to dry eye symptoms, and one was no longer eligible due to pregnancy. Five patients were disenrolled after treatment due to pregnancy at various follow up times. The 12-month follow up visit for the last patient occurred on February 2, 2011. Follow up rates are listed below and include all 143 patients enrolled.
01 M: 99.30%
03M: 97.20%
06M: 93.71%
12M: 89.51%

There has been one adverse event in the entire study. This event was reported in 2009 and was in a subject who experienced acute, non-granulomatous anterior uveitis OS 2 weeks following uncomplicated LASIK. The subject was treated with Prednisolone Acetate 1.0% ophthalmic suspension q2hrs and cyclopentolate 1% ophthalmic solution TID, which resolved the uveitis. The PI felt this was unrelated to the surgery or the study participation.

Results for clinical tests were gathered for all patients in the study and are being analyzed by the statistician. Impression cytology analysis was performed on a subset of normal and dry eye patients. We are now analyzing our data and preparing manuscripts for publication.

KEY RESEARCH ACCOMPLISHMENTS

- Developed a method for measuring multiple second messengers in a single conjunctival sample using bioplex technology.
- EGF and cholinergic agonists cause changes in phosphoproteins ERK and AKT in control, unwounded, mouse conjunctiva, but b-adrenergic agonists alter STAT-3 and perhaps P79S6.
- Two days after wounding the wounded and unwounded conjunctiva respond differently from each other and differently from the control unwounded mice.
- Six days after wounding conjunctival response is lost.
- Two and four weeks after wounding, conjunctival response begins to return.
- Conjunctival cells collected by impression cytology from human subjects do not respond to carbachol and cannot be studied.
- Enrolled 146 subjects for LASIK or PRK completing our patient enrollment.
- Performed analysis of tear film before and after surgery using standardized tests described in our protocol to determine individuals who develop chronic dry eye after refractive surgery.
- Analyzed goblet cell population in a cohort of subjects who did not develop dry eye after surgery compared to a cohort who did. Patients were analyzed for number of filled, empty, and total conjunctival goblet cells.
- Found that low abundance proteins can be analyzed from tears absorbed to Schirmer strips.
- Found that changes in specific inflammatory proteins in tears can be predictive of dry eye and epitheliopathy after refractive surgery.
- Final results from our study are awaiting statistical analysis to determine if subjects who develop dry eye after refractive surgery have a different conjunctival response compared to those who are normal.

REPORTABLE OUTCOMES

Shatos M, Sediq DA, Bower KS, Edwards JD, Peppers L, Coe CD Dartt DA. Goblet Cell Response to Photorefractive Keratectomy (PRK): Effect on Total and Filled GC Number. Invest. Opthalmol. Vis. Sci. 50: E-abstract 574, 2009.

Sack R, Sathe S, Beaton A, Dartt D, Bower K, Coe C, Sediq D, J. Edwards J, Peppers L, Iserovich P. Micro Well Plate Antibody Array Screening of the Pre and Post Refractive Surgical Tears for Biomarkers of Induced Dry Eye. Invest. Opthalmol. Vis. Sci. 50: E-abstract 2547, 2009.

M. Shatos, D.S. Ryan, K.S. Bower, C.D. Coe, L. Peppers, E. Guilbert, J. Doherty, D.A. Dartt. Refractive Surgery Alters the Conjunctival Goblet Cell Population in Individuals Who Develop Dry Eye. Invest. Opthalmol. Vis. Sci. 51: E-abstract 1920, 2010.

D.S. Ryan, M.A. Shatos, K.S. Bower, R.K. Sia, L. Peppers, C.D. Coe, E. Guilbert, R.S. Howard, D.A. Dartt. Normal Goblet Cell (GC) Response After Photorefractive Keratectomy (PRK) and Laser Assisted in situ Keratomileusis (LASIK). Invest. Opthalmol. Vis. Sci. 51: E-abstract 2847, 2010.

C.D. Coe, K.S. Bower, D.S. Ryan, R.A. Sack, P. Pavel Iserovich, M.A. Shatos, D.A. Dartt. Predicting Post-Operative Dry Eye: Multivariate Analysis of Clinical Findings, Tear Proteins, and Goblet Cells. Invest. Opthalmol. Vis. Sci. 51: E-abstract 3364, 2010.

M Shatos, D Ryan, K Bower, C Coe, L Peppers E Guilbert, J Doherty, R Hodges , D Dartt. Refractive Surgery Alters Conjunctival Goblet Cells In Patients Who Develop Dry Eye. Tear Film and Ocular Surface Meeting, Florence Italy, September 2010.

K Bower, M Shatos1, D Ryan2, C Coe2, L Peppers2 E Guilbert1, J Doherty1, R Hodges 1, D Dartt1. Refractive Surgery Alters Conjunctival Goblet Cells In Patients Who Develop Dry Eye. Ophthalmology/Harvard Med Sch, Schepens Eye Research Institute, Boston, MA; 2 Walter Reed Army Medical Center, Washington, DC. Fourth Military Vision Research Symposium. Boston MA September. 2010

CONCLUSIONS

We conclude that in the normal mouse conjunctiva, the epithelial cells differentially respond to growth factors, cholinergic and beta-adrenergic agonists. Corneal wounding that mimics laser refractive surgery changes this response. Unfortunately, human conjunctival cell function cannot be studied on impression cytology samples when samples are frozen before analysis.

We conclude that in individuals who do not develop dry eye after refractive surgery the tear film and conjunctival goblet cell population is unaltered over time. We conclude that immediately after surgery in individuals who develop dry eye, LASIK appears to be more damaging to the ocular surface than PRK, but patients appear to recover by 3 months irrespective of the surgical procedure**.** LASIK decreased the number of filled goblet cells suggesting that LASIK is destroying the nerves that stimulate goblet cell secretion. We also conclude that analysis of low abundance proteins associated with inflammation and corneal wound healing can be used to determine changes in specific proteins associated with dry eye or other complications after refractive surgery.

APPENDIX

1. Sack R, Sathe S, Beaton A, Dartt D, Bower K, Coe C, Sediq D, J. Edwards J, Peppers L, Iserovich P. Micro Well Plate Antibody Array Screening of the Pre and Post Refractive Surgical Tears for Biomarkers of Induced Dry Eye. Invest. Opthalmol. Vis. Sci. 50: E-abstract 2547, 2009.

2. Shatos M, Sediq DA, Bower KS, Edwards JD, Peppers L, Coe CD Dartt DA. Goblet Cell Response to Photorefractive Keratectomy (PRK): Effect on Total and Filled GC Number. Invest. Opthalmol. Vis. Sci. 50: E-abstract 574, 2009.

3. Dartt DA, Shatos M, Sediq D, Edwars J, Peppers L, Coe C, Bower KS. Effect of Refractive Surgery on Conjunctival Goblet Cells in Normal and Dry Eye Subjects. Presented at Fourth Military Refractive Surgery Meeting. January 2010.

4. Shatos M, Sediq D, Bower K, Edwards J, Peppers L, Coe C, Dartt D. Goblet Cell (GC) Response to Photorefractive Keratectomy (PRK): Effect on Total and Filled GC Number. Abstract ARVO 2010.

5. Ryan DS, Shatos M, Bower KS, Sia RK, Peppers L, Coe CD, Howard RS, Dartt DA. Normal Goblet Cell (GC) Response after Photorefractive Keratectomy (PRK) and Laser assisted in situ keratomileusis (LASIK). ARVO 2010 abstract submitted for approval. Control # 10-A-6348-ARVO.

6. Coe CD, Ryan DS, Sack RA, Pavel Iserovich P, Shatos MA, Dartt DA. Predicting Post-operative Dry Eye: Multivariate Analysis of Clinical Findings, Tear Proteins, and Goblet Cells. ARVO 2010 abstract submitted for approval. Control # 10-A-6052-ARVO.

7. M Shatos, D Ryan, K Bower, C Coe, L Peppers E Guilbert, J Doherty, R Hodges , D Dartt. Refractive Surgery Alters Conjunctival Goblet Cells In Patients Who Develop Dry Eye. Tear Film and Ocular Surface Meeting, Florence Italy, September 2010.

Table 1. Alterations in phosphoprotein levels in non-wounded Balb-c control mouse conjunctiva following stimulation with EGF, Carbachol and Isoproterenol using BioRad multiplex assay

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
Phospho ERK	1.35	0.0018 *	1.34	0.0042 *	1.02	0.87
Phospho JNK	1.34	0.053	1.29	0.076	1.14	0.26
Phospho P38	1.06	0.609	0.96	0.73	1.07	0.39
Phospho AKT	1.49	0.044 *	1.22	0.002 *	1.05	0.79
Phospho IkB-alpha	1.41	0.196	1.22	0.07	0.98	0.79
Phospho STAT-3	1.27	0.09	1.32	0.09	1.19	0.012 *
Phospho P70-S6	1.24	0.43	1.30	0.38	0.88	0.18

* $P < 0.05$
N= 7 experiments
Age of mice: 12 weeks old Balb-c mice

Table 2. Alterations in phosphoprotein levels in mouse conjunctiva in the wounded (Rt) eye following stimulation with EGF, Carbachol and Isoproterenol 2 days after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.36	0.0006 *	1.14	0.016 *	0.88	0.07
Phospho JNK	1.44	0.0006 *	1.29	0.0004 *	1.23	0.11
Phospho P38	1.23	0.019 *	1.19	0.22	1.12	0.41
Phospho AKT	1.63	0.39	1.27	0.13	0.87	0.16
Phospho IkB-alpha	1.04	0.31	1.12	0.07	1.03	0.72
Phospho STAT-3	1.23	0.29	1.15	0.21	1.26	0.23
Phospho P70-S6	2.15	0.21	1.53	0.24	1.34	0.39

* P <0.05
N= 5 experiments
Age of mice: 12 weeks old Balb-c mice

Table 3. Alterations in phosphoprotein levels in mouse conjunctiva in the unwounded (left) eye following stimulation with EGF, Carbachol and Isoproterenol 2 days after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.59	0.0001 *	1.23	0.06	1.08	0.46
Phospho JNK	1.18	0.17	1.54	0.02*	1.12	0.16
Phospho P38	1.29	0.04 *	1.28	0.007 *	1.12	0.14
Phospho AKT	1.35	0.21	1.52	0.50	1.66	0.03*
Phospho IkB-alpha	1.17	0.11	1.13	0.01 *	1.08	0.19
Phospho STAT-3	1.13	0.32	1.33	0.04 *	0.92	0.49
Phospho P70-S6	1.24	0.19	1.49	0.009 *	0.89	0.47

* P <0.05
N= 5 experiments
Age of mice: 12 weeks old Balb-c mice

Table 4. Alterations in phosphoprotein levels in mouse conjunctiva in the wounded (right) eye following stimulation with EGF, Carbachol and Isoproterenol 6 days after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.13	0.30	0.84	0.22	1.01	0.86
Phospho JNK	0.97	0.66	0.81	0.40	0.79	0.39
Phospho P38	0.81	0.007 *	0.82	0.10	0.87	0.16
Phospho AKT	0.55	0.04*	0.62	0.20	0.94	0.90
Phospho IkB-alpha	0.89	0.12	1.04	0.38	1.08	0.17
Phospho STAT-3	1.12	0.52	1.01	0.97	1.08	0.46
Phospho P70-S6	0.80	0.53	0.93	0.51	0.72	0.08

* P <0.05
N= 3 experiments
Age of mice: 12-week old Balb-c mice

Table 5. Alterations in phosphoprotein levels in mouse conjunctiva in the unwounded (left) eye following stimulation with EGF, Carbachol and Isoproterenol 6 days after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.74	0.0005*	1.30	0.06	1.13	0.29
Phospho JNK	1.60	0.04*	1.38	0.07	1.07	0.81
Phospho P38	1.60	0.12	1.41	0.10	1.18	0.52
Phospho AKT	1.54	0.15	1.43	0.02*	1.45	0.45
Phospho IkB-alpha	1.63	0.05*	1.69	0.08	1.57	0.36
Phospho STAT-3	1.23	0.09	1.16	0.10	0.90	0.73
Phospho P70-S6	1.50	0.11	1.42	0.27	1.28	0.45

* P <0.05
N= 3 experiments
Age of mice: 12-week old Balb-c mice

Table 6. Alterations in phosphoprotein levels in mouse conjunctiva in the wounded (right) eye following stimulation with EGF, Carbachol and Isoproterenol 2 weeks after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.56	0.03*	1.42	0.002*	1.02	0.89
Phospho JNK	0.97	0.66	1.48	0.08	1.63	0.45
Phospho P38	0.86	0.34	1.22	0.10	0.95	0.68
Phospho AKT	1.36	0.40	1.65	0.38	1.25	0.07
Phospho IkB-alpha	1.06	0.80	1.37	0.26	1.02	0.82
Phospho STAT-3	0.79	0.30	0.86	0.39	0.98	0.93
Phospho P70-S6	1.97	0.54	2.37	0.16	1.11	0.55

* P <0.05
N= 6 experiments
Age of mice: 12-week old Balb-c mice

Table 7. Alterations in phosphoprotein levels in mouse conjunctiva in the unwounded (left) eye following stimulation with EGF, Carbachol and Isoproterenol 2 weeks after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.33	0.17	0.98	0.45	1.10	0.22
Phospho JNK	1.77	0.17	0.72	0.09	0.88	0.45
Phospho P38	1.29	0.09	0.70	0.02*	0.94	0.51
Phospho AKT	1.35	0.40	0.91	0.55	0.97	0.62
Phospho IkB-alpha	1.17	0.06	0.80	0.23	1.12	0.34
Phospho STAT-3	1.17	0.41	0.73	0.08	0.97	0.87
Phospho P70-S6	1.94	0.11	0.91	0.75	1.01	0.96

* P <0.05
N= 6 experiments
Age of mice: 12-week old Balb-c mice

Table 8. Alterations in phosphoprotein levels in mouse conjunctiva in the wounded (right) eye following stimulation with EGF, Carbachol and Isoproterenol 4 weeks after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.63	0.0006*	1.21	0.11	1.07	0.62
Phospho JNK	1.30	0.39	1.28	0.13	1.21	0.49
Phospho P38	1.14	0.24	1.05	0.66	1.24	0.20
Phospho AKT	1.87	0.33	1.11	0.68	0.97	0.91
Phospho IkB-alpha	1.28	0.23	1.17	0.21	1.16	0.33
Phospho STAT-3	0.94	0.77	1.08	0.38	1.18	0.21
Phospho P70-S6	1.14	0.61	1.05	0.68	0.99	0.96

* P <0.05
N= 6 experiments
Age of mice: 12-week old Balb-c mice

Table 9. Alterations in phosphoprotein levels in mouse conjunctiva in the unwounded (left) eye following stimulation with EGF, Carbachol and Isoproterenol 4 weeks after corneal wounding

	EGF		Carbachol		Isoproterenol	
	Fold increase over basal	P value	Fold increase over basal	P value	Fold increase over basal	P value
PERK	1.74	0.05*	1.51	0.15	1.24	0.39
Phospho JNK	1.37	0.25	1.34	0.13	1.39	0.11
Phospho P38	1.53	0.15	1.31	0.35	1.48	0.17
Phospho AKT	1.52	0.21	1.72	0.09	1.56	0.23
Phospho IkB-alpha	1.31	0.34	1.34	0.34	1.64	0.21
Phospho STAT-3	1.49	0.19	1.14	0.51	1.51	0.09
Phospho P70-S6	2.76	0.20	1.58	0.36	1.85	0.28

* P <0.05
N= 6 experiments
Age of mice: 12-week old Balb-c mice

Table 10. Mouse conjunctival stimulation experiments for multiplex analysis of phosphoproteins

Experiment type	No. of experiments done	No. of experiments analyzed by multiplex	No. of experiments to be analyzed by multiplex
Unwounded	7	7	0
2 day post-wound	5	5	0
6 day post-wound	4	3	1 (lost)
2 week post-wound	6	6	0
4 week post-wound	6	6	0

Micro Well Plate Antibody Array Screening of the Pre and Post Refractive Surgical Tears for Biomarkers of Induced Dry Eye

Authors: R. Sack[1], S. Sathe[1], A. Beaton[1], D. Dartt[2], K. Bower[3], C. Coe[3], D. Sediq[3], J. Edwards[3], L. Peppers[3], P. Iserovich[1].
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Purpose: To refine array technology to allow the quantitative screening of tears for biomarkers that might predict risk of dry eye (DE) and epithelialopathy induced by refractive surgery.

Methods: Commercial and custom array kits were extensively modified to increase sensitivity and eliminate a previously confounding tear matrix effect (Sack etal. Ex. Eye Res. 2007) and thereby allow the assay of 40 + low abundance protein (LAP) that modulate inflammation and wound healing. Tear samples were collected so far from 67 patients before and after refractive surgery (LASIK and PRK) with Schirmer strips (SS). Samples analyzed came from 9 individuals who developed bilateral decreases in SS values from ~30 to10-0 mm accompanied in 1 instance with epithelialopathy and matched sets of samples from 9 “controls” who exhibited negligible decrease in SS values. The bulb of each SS was extracted in a proprietary buffer and aliquots assayed with a 16 plex cytokine array and several 9 plex arrays designed to provide redundant assays for several proteins.

Results: Sufficient levels of ~14-18 LAPs were present in most extracts to allow quantification. These include cytokines, chemokines, growth factors, angiogenic modulators and proteases and inhibitors. The concentrations of many of these proteins (both pro and anti-inflammatory) increased in the vast majority of the post surgical samples. 4/9 of the pre-surgical samples from the DE population exhibited an unique LAP profile indicative of markedly lower concentrations of 1, sometimes 2 LAPs that serve to down-regulate inflammation. This absence was most extreme in bi-lateral pre-surgical samples obtained from an individual who developed severe DE and epithelialopathy. This difference was not apparent in the post surgical samples. A similar profile has yet to be observed in the assay of comparable samples obtained from individuals with a wide range of active ocular surfaces diseases.

Conclusions: Micro well arrays can be used for quantitative assay of LAPs in tears. Results suggest that ~4 % of the population may have a LAP profile that is indicative of a reduced capacity to handle short-term inflammatory stress. This protein loss may be a risk factor contributing to post-surgical DE and epithelialopathy.

Goblet Cell Response to Photorefractive Keratectomy: Effect on Total and Filled Goblet Cell Number

Authors: M. Shatos[1], D. A. Sediq[2], K. S. Bower[2], J. D. Edwards[2], L. Peppers[2], C. D. Coe[2] and D. Dartt[1]
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Purpose: To determine if filled GC represent the total conjunctival GC population and if PRK alters the number of these cells.

Methods: Impression cytology samples (ICS) were taken from the superior and temporal conjunctivae of 8 patients (3 males, 5 females; average age 30 yr) before and 1M after PRK. Tear film status was evaluated by Schirmer test (ST) without anesthesia, tear breakup time (TBUT), and McMonnies Questionnaire (MQ). ICS were transferred to glass slides and stained with anti-keratin 7 (K7), marks GC bodies; Helix pomatia agglutinin (HPA), marks GC secretory product; and DAPI, marks cell nuclei. Five areas were counted for each sample. The total number of cells was determined by counting DAPI stained nuclei, total number of GC(filled and empty) by determining the number of DAPI stained nuclei positive for K7, and number of filled GC by measuring the number of DAPI stained nuclei positive for both K7 and HPA.

Results: Mean spherical equivalent pre-op was -2.99+/-0.35 diopters. Average ablation depth for the 8 patients was 48.4+/-5.2µ. ST value was 21.3+/-2.7 mm before surgery and decreased to 16.4+/-2.5 mm 1M post-op. Average TBUT significantly decreased from 20.1+/-2.4 sec pre-op to 11.5+/-1.5 sec at 1M post-op. Score on MQ increased from 5.9+/-1.1 pre-op to 8.0+/-1.7 post-op. Total number of GC did not differ from the number of filled GC in the superior conjunctiva, being 146+/-29 and 121+/-25 respectively pre-op and 61+/-15 and 52+/-14 post-op. Similar results were obtained in the temporal ICS. Both total number and number of filled GC in the superior, but not temporal, conjunctiva significantly decreased post-op compared to pre-op. Total number of cells decreased from 146+/-29 to 61+/-15; number of filled GC decreased from 121+/-25 to 52+/-14.

Conclusions: There is not a significant population of unfilled GC in the conjunctiva. PRK decreased the GC population in the superior, but not temporal, conjunctiva accompanied by changes consistent with surgically-induced dry eye.

Goblet Cell Response to Photorefractive Keratectomy: Effect on Total and Filled Goblet Cell Number

Authors: M. Shatos1, D.A. Sediq2, K.S. Bower2, J.D. Edwards2, L. Peppers2, C.D. Coe2, D. Dartt1.
1Ophthal/Harvard Med Sch, Schepens Eye Research Institute, Boston, MA; 2Ophthalmology, Walter Reed Army Medical Center, Washington, DC.

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Normal Goblet Cell (GC) Response After Photorefractive Keratectomy (PRK) and Laser Assisted *in situ* Keratomileusis (LASIK)

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Purpose: To examine the post-op GC response after uncomplicated PRK and LASIK.

Methods: Dry eye (DE) questionnaire, Schirmer test, tear breakup time, and Rose Bengal staining were tested pre- and post-op. Patients whose post-op course was complicated by DE were excluded. Impression cytology samples (ICS) were taken from the superior and temporal conjunctivae of 12 PRK (8F, 4M) and 8 LASIK patients (3F, 5M) pre-op and at 1W, and 1 and

3M post-op. ICS were fixed on glass slides and stained with three indicators: DAPI to mark cell nuclei to determine the total number of cells (GC and squamous epithelial cells); with anti-keratin 7 (K7) to mark GC bodies to determine the percentage of cells which were GC (% GC); and with K7 and helix pomatia agglutinin to mark GC secretory product to determine the percentage of GC which were filled (% Filled GC). (Figure) Five random areas were selected as representative fields of the total slide. Results were compared using RM-ANOVA with $p<0.05$ considered significant.

Results: Mean age±SD of the PRK group was 28.5±5.1 and LASIK was 32.8±5.1 years (p=0.18). Mean ablation depth of PRK was 55.40±18.10μm and LASIK was 56.01±17.85μm (p=0.91). The % GC did not change significantly over time in either the PRK group [46.2 ±27.6% pre, 34.8±22.7% 1W, 41.8±25.9% 1M, 45.5±21.9% 3M (p=0.43)] or the LASIK group [35.6 ±20.6% pre, 22.8±19.8% 1W, 17.4±14.9% 1M, 36.5±22.8% 3M (p=0.18)]. The % Filled GC did not change significantly over time in either the PRK group:64.7±35.4% pre, 80.1±17.9% 1W, 76.6±20.9% 1M, 68.0±33.2% 3M (p=0.29) or the LASIK group: 55.3 ±36.9% pre, 74.4±22.1% 1W, 70.3±27.6% 1M, 64.0±27.5% 3M (p=0.24).

Conclusions: Preliminary results indicate that neither the percentage of GC nor the proportion of GC which are filled changes significantly over time after either PRK or LASIK in patients uncomplicated by post-op DE. GC response in post-surgical DE patients is currently under investigation**.**

Support: Department of Defense Grant W81XWH-04-2-0008

Predicting Post-Operative Dry Eye: Multivariate Analysis of Clinical Findings, Tear Proteins, and Goblet Cells

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Purpose: To examine the significance of subjective symptoms, the clinical examination, conjunctival goblet cells, and inflammatory tear proteins and cytokines in predicting post-operative dry eye in refractive surgery patients.

Methods: A prospective, non-randomized, multicenter study comparing the effect of LASIK and PRK on the clinical findings of cochet-bonnet aesthesiometry, tear break up time (TBUT), rose Bengal staining (RB), videokeratoscopy surface indices, percent of filled goblet cells (GC), 16 cytokines, 7 matrix metalloproteinases, 2 matrix metalloproteinases inhibitors, and subjective sxs of dry eye quantified using the McMonnies questionnaire. Discriminant analysis

was conducted at 1 week (1W), 1 month (1M), and 3 months (3M) to determine if significant differences existed across the predictor variables of two groups: subjects with and without postoperative dry eye.

Results: Seventy-two eyes underwent either PRK(n=39) or intralase LASIK (n=33). At one week post-op, only MMP10 (Wilks' lambda=0.461) and IL1α(Wilks' lambda=0.302) were significant predictor variables. At one month, percent filled of goblet cells (WL=.791), Rose Bengal Staining (WL=0.617), and age (WL=0.533) were different across the two groups. At 3M, McMonnies questionnaire (WL=0.724), Rose Bengal staining (WL=0.565), Schirmer's with anesthesia (WL=0.478), and age (WL=0.413) were significantly different across the two groups. The level of tear proteins were not significantly different across the two groups at either 1M or 3M and at no time point did videokeratoscopy surface indices distinguish between the dry eye group and non-dry eye group.

Conclusions: In the immediate post-operative period, there are changes in both the magnitude of tear proteins and goblet cells that contribute to dry eye. Beginning at 1M, the clinical exam and patient demographics are the best predictors of post-operative dry eye.

REFRACTIVE SURGERY ALTERS CONJUNCTIVAL GOBLET CELLS IN PATIENTS WHO DEVELOP DRY EYE

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Objective: To determine if conjunctival goblet cell (GC) profiles differ in persons who develop dry eye (DE) after laser-assisted in situ keratomileusis (LASIK) or photorefractive keratectomy (PRK).

Method: Tear film status was evaluated by slit lamp biomicroscopy, Schirmer test, tear breakup time and McMonnies DE questionnaire. Patients with clinically significant DE were enrolled in this study. Impression cytology samples (ICS) were taken from superior and temporal conjunctivae pre-, at 1w, 1m and 3m post-op. ICS on membranes were stained with anti cytokeratin-7 (K7) to identify GC, Helix pomatia agglutinin (HPA) for GC secretory product, and DAPI for cell nuclei. Five random fields were counted per sample. Total cell number was determined by counting DAPI stained nuclei; unfilled GC number by K7 positive cells, and filled GC by K7 cells with HPA. Statistics were performed using student's t-test with $p<0.05$as significant.

Result: 19 post- DE patients were studied [9 LASIK, (3M, 6 F, average.age 33y); 10 PRK (6M, 4F, average age 27 y)].The %total GC (filled plus unfilled) decreased at 1w in both PRK (27±5) and LASIK (35±7) when compared to their respective pre- levels of 33±4 and 51±7, but recovered at 3M. For LASIK but not PRK, when the total number of GC was set to 100%, the

% filled GC significantly increased at 3M post- (69±11) compared to pre- (40±10). LASIK but not PRK,, decreased the number of empty GCat 3m (31±11) to pre- values of (60±11).

Conclusion : In individuals who develop dry eye after refractive surgery**,** LASIK appears more damaging to the ocular surface than PRK perhaps by destroying corneal sensory nerves, and preventing GC secretion.

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